PB89-237424

Development of a Protocol to Assess Reproductive Effects of Toxicants in the Rat

(U.S.) Health Effects Research Lab., Research Triangle Park, NC

1988

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TECHNICAL REPORT DATA

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1. REPORT NO.	2.	3. PB89-237424
EPA/600/J-88/342		

4. TITLE AND SUBTITLE
THE DEVELOPMENT OF A PROTOCOL TO ASSESS REPRODUCTIVE EFFECTS OF TOXICANTS IN THE RAT

5. REPORT DATE

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Environmental Protection Agency (MD-72)
Health Effects Research Laboratory
Developmental & Cellular Toxicology Division
Reproductive Toxicology Branch, RTP, NC

10. PROGRAM ELEMENT NO.
ACSL1A

11. CONTRACT/GRANT NO.
In House

12. SPONSORING AGENCY NAME AND ADDRESS
Health Effects Research Laboratory/RTP, NC
Office of Research & Development
US EPA, RTP, NC 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA 600/11

15. SUPPLEMENTARY NOTES
Presented: Reproductive Toxicology, vol. 2, pp. 281-287, 1988

16. ABSTRACT

The determination that a chemical poses a reproductive risk to man typically relies upon fertility studies using rodents. However, fertility in rodents is often difficult to disrupt and more sensitive indicators of reproductive function should be incuded in the risk assessment process. The present discussion compares the sensitivity of fertility to other endpoints following exposure to known reproductive toxicants. In our studies rats were dosed from weaning, through puberty, gestation, and lactation. The reproductive function of the male, the female and the offspring was assessed. The effects of methoxychlor, carbendazim, dibutyl phthalate and the lindane protocol are discussed and compared to fertility. For each compound a ratio (SR=sensitivity ratio) of the lowest effect level (LEL) for infertility or reduced fecundity to the LEL for the most sensitive physiological endpoint was calculated. The SR ratio should be large when a compound produces effects over wide range of doses, but should equal unity when the dose-response curve is very steep. For methoxychlor, which block implantation, pubertal landmarks and estrous cyclicity provided rapid and sensitive indicators of the subsequent reproductive failure. The SR=8 (100/12) for methoxychlor using data from the females. In contrast, DBP and MBC directly altered testicular function, and for these compounds sperm and testicular measures provided sensitive indicators of toxicity. The SR for MBC was 2 (100/50), while DBP had a SR of 1 (250/250). In the lindane study, fertility was not reduced but most of the pups (F1) died shortly after birth. The SR for lindane is equal to 0.5 (10/20). At 20mg/kg the treated-females were larger and their estrous cycles were erratic. The data from these studies indicate that no single endpoint will consistently be the most sensitive indicator of reproductive toxicity. Studies must include a number of well validated endpoints that provide a comprehensive assessment of the entire reproductive system of the male and female.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
U.S. DEPARTMENT OF COMMERCE
NATIONAL TECHNICAL INFORMATION SERVICE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	9
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA/600/J-88/342

Reproductive Toxicology, Vol. 2, pp. 281–287, 1988
Printed in the U.S.A.

THE DEVELOPMENT OF A PROTOCOL TO ASSESS REPRODUCTIVE EFFECTS OF TOXICANTS IN THE RAT

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Abstract — The determination that a chemical poses a reproductive risk to man typically relies upon fertility studies using rodents. However, fertility in rodents is often difficult to disrupt and more sensitive indicators of reproductive function should be included in the risk assessment process. The present discussion compares the sensitivity of fertility to other endpoints following exposure to known reproductive toxicants. In our studies rats were dosed from weaning through puberty, gestation, and lactation. The reproductive function of the male, the female, and the offspring was assessed. The effects of methoxychlor, carbendazim (MBC), dibutyl phthalate (DBP), and lindane are discussed and compared to fertility. For each compound a ratio (SR = sensitivity ratio) of the lowest effect level (LEL) for infertility or reduced fecundity to the LEL for the most sensitive physiologic endpoint was calculated. The SR should be large when a compound produces effects over a wide range of doses, but should equal unity when the dose–response curve is very steep. For methoxychlor, which blocked implantation, pubertal landmarks and estrous cyclicity provided rapid and sensitive indicators of the subsequent reproductive failure. The SR = 8 (100/12) for methoxychlor using data from females. In contrast, DBP and MBC directly altered testicular function, and for these compounds, sperm and testicular measures provided sensitive indicators of toxicity. The SR for MBC was 2 (100/50), while DBP had a SR of 1 (500/500). In the lindane study, fertility was not reduced but most of the pups (F1) died shortly after birth. The SR for lindane is equal to 0.5 (10/20). At 20 mg/kg the treated females were larger and their estrous cycles were erratic. The data from these studies indicate that no single endpoint will consistently be the most sensitive indicator of reproductive toxicity. Studies must include a number of well validated endpoints that provide a comprehensive assessment of the entire reproductive system of the male and female.

Key Words: Fertility, Rat, Methoxychlor, Carbendazim, Dibutyl phthalate, Lindane, Measures, Sperm and Endocrine.

INTRODUCTION

Presently, no observed effect levels (NOELs) for reproductive toxicity are set using data on fertility, litter size, and litter viability. These endpoints are often insensitive indices of reproductive health. For example, a number of studies, using both male rats and mice, have found that fertility is normal even when sperm counts are reduced by 90% (1). In another study, male mice with severely depleted numbers of germ cells, induced by in utero exposure to a dye, reproduced continuously at normal levels over an 11-month period (2). In the female, so few data exist for endpoints other than fertility that it is impossible to determine if adverse effects occur at doses below those that alter fertility. Thus, it may be appropriate to include additional endpoints of reproductive function in fertility studies. While some consideration has been given to including more sophisticated assessments of testicular histology (3,4) and sperm function (5,6) in test protocols, effects on other reproductive organs in the male (such as pituitary and endocrine function) and additional reproductive measures in females (such as estrous cyclicity) have received little consideration.

PROTOCOL USED TO ASSESS REPRODUCTIVE EFFECTS

We have developed a protocol that measures puberty and fertility, as well as morphologic and physiologic indices of reproductive status (7). The

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Protocol to Assess Reproductive Effects of Toxicants in the Rat

Initiate Daily Dosing

Weaning d 21	Puberty	Young Adulthood	Breeding	Necropsy Males	Gestation/ Lactation	Necropsy Females	Offspring
Experiment 1 **Methoxychlor** (25, 50, 100, or 200 mg/kg/d)	**Females** **d 21–on** • Age at Vaginal Opening • Age at First Estrus • Age at Onset of Cyclicity • Daily Vaginal Smears		Day 80–90	Day 100–105		Day 25 Postpartum	0 and 50 mg/kg **Continous Breeding** 11 Months
Experiment 2 **Carbendazim** (50, 100, 200, or 400 mg/kg/d)							0, 100, and 200 mg/kg **Continous Breeding** 7 Months
Experiment 3 **Dibutyl Phthalate** (250, 500 or 1000 mg/kg/d)	**Males** **d 21–on** • Age at Preputial Separation						**No Offspring**
Experiment 4 **Lindane** (10 or 20 mg/kg/d)							**No Offspring**

Fig. 1. The protocol used in the present investigation to compare multiple endpoints of reproductive function in the male and female rat to infertility.

present study was designed to develop dose–response data for a number of different endpoints following subchronic exposure to known reproductive toxicants during the pubertal, young adult, gestational, and lactational stages of life (Figure 1). We propose that the identification of the susceptible population, the target organ, and the dose–effect relationships for more sensitive reproductive endpoints, as compared to fertility alone, will allow for a more scientifically based extrapolation of the effects seen in studies using rats to potential effects in man.

The present discussion describes the reproductive alterations induced by subchronic administration of known toxicants. Methoxychlor is a weakly estrogenic pesticide that blocks implantation (rats were dosed with 25, 50, 100, or 200 mg/kg/d). Carbendazim (MBC) is a fungicide that inhibits microtubule function and causes testicular toxicity and teratogenicity (MBC doses of 50, 100, 200, and 400 mg/kg/d were used). Dibutyl phthalate is a plasticizer that also alters Sertoli cell function (rats were dosed with DBP at 250, 500, or 1000 mg/kg/d). Lindane is a pesticide that inhibits the effects of estrogen on the uterus (doses of 10 and 20 mg/kg/d were used) (Figure 1).

Morphological changes associated with puberty were measured (the ages at the opening of the vagina, the first estrous smear, and the onset of regular estrous cycles), and vaginal estrous cycles were monitored until parturition. After the F1 pups were weaned the females were killed and necropsied, body, pituitary, ovarian, liver, kidney, and adrenal weights were measured, and the number of implantation sites were counted. The ovaries and uteri were saved for histopathologic examination. The viability and growth of the offspring were monitored, and when there were sufficient numbers of viable offspring, their fertility and fecundity were monitored for at least six months.

Growth and preputial separation were measured in males because the latter is a noninvasive androgen-dependent index of puberty (8). The males were necropsied at about 100 days of age; serum was collected; testosterone, androgen binding protein, FSH, LH, prolactin, and TSH were measured; and body, testes, liver, kidney, adrenal (an estrogen-stimulated gland), seminal vesicle (an androgen-sensitive tissue), epididymal, and pituitary weights were taken. One testis was saved for histological evaluation. At this time a sample of caudal sperm was taken, the percent of motile sperm was estimated, and cellular debris, clumps of immotile sperm, and abnormal sperm were noted if present in the sample. The other testis, the caput epididymis, and the pituitary were utilized for hormonal content. Testicular interstitial fluid (IF) was collected (9) and this testis was used for hCG-

Effects of Reproductive Toxicants on the Male Rat

	Methoxychlor				Carbendazim						Dibutyl Phthalate				Lindane	
	25	50	100	200	50	100	200F	200IF	400F	400IF	250	500F	500IF	1000IF	10	20
Body Weight at Necropsy (g)		↓	↓↓	↓↓								↓		↓		
Liver Weight (g)			↓↓	↓↓												↑↑
Kidney Weight (g)			↓↓	↓↓											↑↑	↑↑
Adrenal Weight (mg)			↑↑	↑↑												
Seminal Vesicle Weight (g)		↓	↓↓	↓↓												
Age at Preputial Separation (g)			↓↓	↓↓												
Weight at Preputial Separation (g)				↑↑												
Testicular Measures																
Testis Weight (g)				↓↓			↓	↓↓	↓↓	↓↓		↓	↓↓	↓↓		
Interstitial Fluid (IF) Weight (g)																
Seminiferous Tubule Fluid (SNF) Weight (g)													↓	↓	ND	ND
IF Testosterone			↓↓					↑↑		↑↑				↑		
IF ABP								↑↑		↑↑					ND	ND
SNF Testosterone															ND	ND
SNF ABP								↑↑		↑↑					ND	ND
Serum Testosterone				↓												
Serum ABP				↓↓					↑	↑			↓↓	↓↓	ND	ND
hCG Stimulated Testosterone																
0 Hour			↓↓	↓												
1 Hour			↓						↑	↑↑				↑↑		
2 Hour				↓			↑	↑↑	↑↑	↑↑				↑↑		
3 Hour				↓↓			↑	↑↑	↑↑	↑↑				↑↑	ND	ND

INC —Incomplete data.
ND —No data.
↑ —significantly increased p<0.05.
↑↑ —significantly increased p<0.001.
↓ —significantly decreased p<0.05.
↓↓ —significantly decreased p<0.001.
IF —Infertile Males.
F —Fertile Males.

Fig. 2. The effects of methoxychlor, carbendazim, dibutyl phthalate, or lindane on organ weights and testicular endpoints in the male rat.

stimulated testosterone synthesis in vitro and then for sonication-resistant spermatid head count (10). The cauda epididymides were used for a quantitative assessment of progressive sperm motility, sperm morphology, and total sperm content. The percentage of progressively motile sperm was measured (5). In addition, 200 sperm heads, or intact sperm, per animal were categorized as normal intact cells with no abnormalities, or as abnormal.

ASSESSMENT OF EXPOSURE TO METHOXYCHLOR

Summaries of the reproductive effects following exposure to methoxychlor are shown in Figures 2 and 3 for males and in Figure 4 for females. The weakly estrogenic pesticide methoxychlor accelerated vaginal opening, altered estrous cyclicity, inhibited ovarian luteal function, and blocked implantation (11). The acceleration of vaginal opening followed by persistent estrus is a clear demonstration of the estrogencity of methoxychlor since this is a standard bioassay for estrogens. This effect was apparent after four days of treatment even at the lowest dose, and a subsequent study demonstrated an LEL (lowest effect level in mg/kg/d) of 12 for this endpoint. Although the high dose group females mated, none of them became pregnant due to failure of implantation. Ovarian weight was reduced, and the ovaries of 5/8 of these females had no visible corpora lutea. The sensitivity ratio (SR = the LEL for fertility or fecundity/the LEL for the most sensitive physiologic endpoint) for methoxychlor was about 8 (100/12).

Perinatal administration of methoxychlor to the dam altered sex differentiation in the female fetus

Effects of Reproductive Toxicants on the Male Rat (continued)

	Methoxychlor				Carbendazim						Dibutyl Phthalate				Lindane	
	25	50	100	200	50	100	200F	200IF	400F	400IF	250	500F	500IF	1000IF	10	20
Epididymal Measures																
Caudal Epididymal Weight (mg)		↓	↓↓	↓↓				↓↓	↓↓	↓↓		↓	↓↓	↓↓		
Caput Epididymis Weight (mg)								↓↓	↓	↓↓		↓	↓↓	↓↓		
Epididymal Testosterone			↓↓	↓↓											ND	ND
Epididymal ABP														↓↓	ND	ND
Sperm Measures																
Caudal Epididymal Sperm Count ($\times 10^6$)		↓↓	↓↓	↓↓	↓	↓	↓↓	↓↓	↓↓	↓↓			↓↓	↓↓		
Sperm Motility (%)																
Testicular Sperm Head Count ($\times 10^6$)							↓↓	↓↓	↓↓	↓↓			↓↓	↓↓		
Pituitary Measures																
Pituitary Weight (mg)			↓↓	↓↓												
Pituitary LH								↑↑		↑↑				↑↑	INC	INC
Pituitary FSH			↑↑	↑↑				↑↑		↑↑					INC	INC
Pituitary Prolactin	↑↑	↑↑	↑↑	↑↑											INC	INC
Pituitary TSH			↑	↑											ND	ND
Serum LH													↑↑	↑↑	INC	INC
Serum FSH								↑↑		↑↑			↑↑	↑↑	INC	INC
Serum Prolactin															INC	INC
Serum TSH			↓↓	↓↓											ND	ND

INC —Incomplete data.
ND —No data.
↑ —significantly increased $p<0.05$.
↑↑ —significantly increased $p<0.001$.
↓ —significantly decreased $p<0.05$.
↓↓ —significantly decreased $p<0.001$.
IF —Infertile Males.
F —Fertile Males.

Fig. 3. The effects of methoxychlor, carbendazim, dibutyl phthalate or lindane on epididymal, sperm, and pituitary measures in the male rat.

such that fecundity was reduced. These females were defeminized by perinatal exposure to this weak estrogen and consequently they produced 50% fewer offspring than did controls. In addition, a higher percentage of the treated females were acyclic during middle age. No significant abnormalities were noted in the male offspring.

In the male, methoxychlor inhibited somatic growth (even at 25 mg/kg) and accessory gland weight, pituitary and serum prolactin levels were elevated, and testicular Leydig cell function was suppressed. The highest dose level used here (200 mg/kg/d) resulted in a minimal alteration of spermatogenesis. These males were fertile even though epididymal sperm storage was markedly reduced. Preputial separation (PPS) was delayed by methoxychlor treatment in the 100 and 200 mg/kg/d dose groups. These doses also inhibited testosterone production. The males with the longest delay in PPS also displayed the most severe alterations of epididymal function and spermatogenesis. In a lower dose group (50 mg/kg/d), cauda epididymal weight, sperm reserves, and seminal vesicle weight were reduced. Testicular lesions were not detected at any dose.

Growth was retarded in the male at all dose levels, weight being reduced by 23% and 32% at 100 and 200 mg/kg/d, respectively. Pharmacologic doses of estrogens retard skeletal growth in both man and rats. In rats estrogens also reduce growth through effects on food consumption and metabolism (12). We propose that an evaluation of the reproductive toxicity of estrogenic compounds like methoxychlor (including estradiol and DES) using a multigenerational test protocol, would detect infertility only at "toxic" dosage levels.

In addition, some estrogens are teratogenic in humans and rodents, and some, like DES, are transplacental carcinogenics as well. It is critical, therefore, in the assessment of the reproductive risk

Effects of Reproductive Toxicants on the Female Rat

	Methoxychlor				Carbendazim				Dibutyl Phthalate			Lindane	
	25	50	100	200	50	100	200	400	250	500	1000	10	20
Age at Vaginal Opening	↓↓	↓↓	↓↓	↓↓									
Weight at Vaginal Opening	↓↓	↓↓	↓↓	↓↓									
Age at First Cycle	↓↓			↑↑									
Age at First Estrus	↓↓	↓↓	↓↓	↓↓									
Percent Normal Cyclicity		↓	↓↓	↓↓									↓
Final Body Weight			↓↓	↓↓									↑↑
Liver Weight				↓↓									↑↑
Kidney Weight				↓									↑
Adrenal Weight			↑	↑									
Pituitary Weight			↓	↓↓									
Paired Ovary Weight			↓	↓↓									
Uterus Weight													
Number of Implantation Sites			↓	↓			↓	↓		↓	↓		
Number of Resorptions						↑	↑	↑					
Number of Fetuses						↓	↓	↓					
Fertility			↓↓	↓↓			↓	↓					
Litter Size			↓↓	↓↓		↓	↓	↓			↓↓	↓↓	↓↓
Malformations in Offspring						↑	↑	↑					
Fertility of Offspring	ND	↓↓	ND	ND		ND	ND	ND					
Running Wheel Activity	ND	ND	ND	↑↑	ND	ND	ND	ND					↓
Ovarian Histology Effects			↑↑	↑↑									
Uterine Histology Effects				↑↑									

ND —No data.
↑ —significantly increased $p<0.05$.
↑↑ —significantly increased $p<0.001$.
↓ —significantly decreased $p<0.05$.
↓↓ —significantly decreased $p<0.001$.

Fig. 4. The effects of methoxychlor, carbendazim, dibutyl phthalate, or lindane on puberty, estrous cyclicity, organ weights, and fertility in the male rat and their offspring.

to humans that test protocols detect estrogenicitiy as well as infertility.

ASSESSMENT OF EXPOSURE TO CARBENDAZIM (MBC)

The results of the studies using dibutyl phthalate (DBP) and the fungicide carbendazim (MBC) that directly alter testicular function are quite different from those previously described for methoxychlor. In the MBC study, rats were dosed from weaning through breeding with 50, 100, 200, or 400 mg/kg/d. Although all of the males treated with MBC at 50 and 100 mg/kg/d were fertile, a number of reproductive changes were noted in the parameters measured. Debris was detected in the caudal sperm sample of most of these males, and caudal sperm reserves were reduced as well. In the 200 and 400 mg/kg/d dosage groups the males were more severely affected and about half of them were infertile. The fertile males had reduced caudal sperm reserves (down by 77%), debris and broken sperm were present in the caudal sample, testicular sperm head counts were reduced (by 50%), and testis weights were reduced (by 25%) (13). The testicular and pituitary hormones were not affected. The second portion of males from the 200 and 400 mg/kg/d dosage groups, which were infertile, had more severe alterations of sperm and endocrine measures as compared to the fertile males at the same doses. In these males testicular sperm head counts were reduced by 90%, cauda epididymal sperm reserves

were reduced to 1% of control, testis weights were down by 50%, serum LH and FSH were increased (14), and a number of endocrine indices of Sertoli and Leydig cell function were altered. None of the endpoints of reproductive function in the female rat were altered by MBC treatment. MBC administration did induce malformations in offspring (hydrocephaly and kinky tails) at 100 mg/kg/d, while higher doses caused fetal death. The SR for MBC was 2 (100/50).

ASSESSMENT OF EXPOSURE TO DIBUTYL PHTHALATE

In the DBP study, male rats were dosed as previously described at 250, 500, or 1000 mg/kg/d. The 250 mg/kg/d dose was a NOEL and the SR was equal to 1 (250/250). Three of 8 males at 500 and 8/8 males at 1000 mg/kg/d were infertile. In the five fertile males treated with DBP at 500 mg/kg/d, testis (down 15%), epididymal weights (down 15%), and caudal sperm reserves (reduced by 20%) were significantly reduced. Testis and cauda epididymal weights, caudal sperm reserves, and testicular sperm counts were dramatically reduced in the infertile males. Serum LH and FSH were elevated and serum ABP was reduced in these infertile, germ cell depleted males. hCG-stimulated testosterone synthesis was enhanced in only the highest dosage group.

DBP-treated females had normal estrous cycles and pubertal landmarks. Fetal death occurred in dams treated with the highest dose (1000 mg/kg/d). It is not clear at this time if this effect represents a fetal or a maternal effect.

ASSESSMENT OF EXPOSURE TO LINDANE

The effects of lindane (10 and 20 mg/kg/d) on reproductive function were both surprising and dramatic. Reproductive development of the male was not affected at any dose. Although the females in the high dose group (20 mg/kg/d) had irregular estrous cycles, these animals were fertile, produced normal litter sizes, and the pups were not malformed. However, all of the offspring in the 20 mg/kg dosage group and some of those in the lower dose group (10 mg/kg/d) died shortly after birth. Preliminary observations suggest that this was due to a maternal factor, related either to a failure of lactation or the lack of maternal care. The SR for lindane was 0.5 (10/20).

SUMMARY

The studies using MBC and DBP demonstrate that for those compounds that directly alter testicular function, infertility was highly correlated with testicular sperm production, elevated serum LH and FSH, and alterations of Leydig cell function. In these studies testicular sperm head counts, cauda epididymal sperm reserves, and testis weights were reduced in treated males that were not infertile. In general, dramatic reductions in sperm production were required to produce infertility in the male rat. These compounds produced fairly steep dose-effect curves as the SR for MBC and DBP were 2 and 1 respectively.

While sperm and testicular measures were sensitive to the testicular toxicants MBC and DBP, treatment with the estrogenic pesticide methoxychlor produced a different profile of alterations. The SR was 8, indicating that the dose-effect relationship for methoxychlor is not nearly as steep as those for MBC and DBP. Pubertal landmarks, estrous cyclicity, and endocrine parameters were sensitive endpoints, while sperm motility and testicular sperm production were not. The data from these studies indicate that no single endpoint will consistently be the most sensitive indicator of reproductive toxicity. Studies must include a number of well validated endpoints that provide comprehensive assessment of the entire reproductive system of the male and female. It is also clear from the results of the lindane study (SR = 0.5) that it would be in error to eliminate breeding trials from reproductive studies.

Acknowledgements — We would like to acknowledge the extremely valuable contributions of L. Strader, K. McElroy and J. Hein for technical assistance and Ms. G. Drake who helped edit and type the manuscript.

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